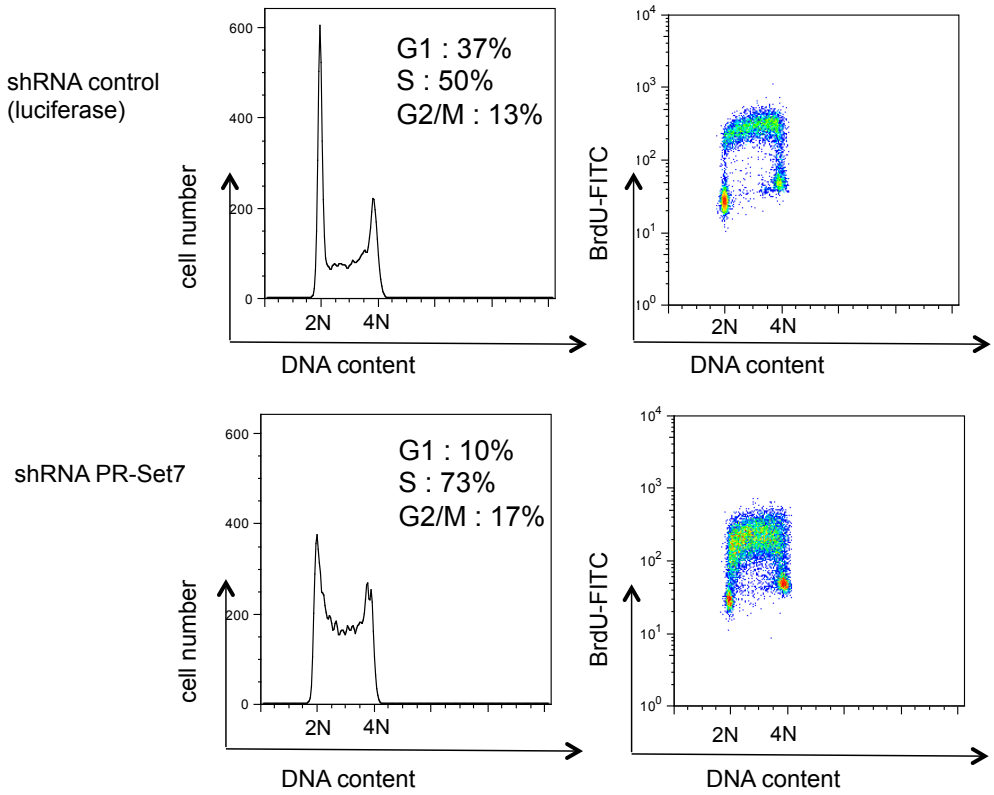
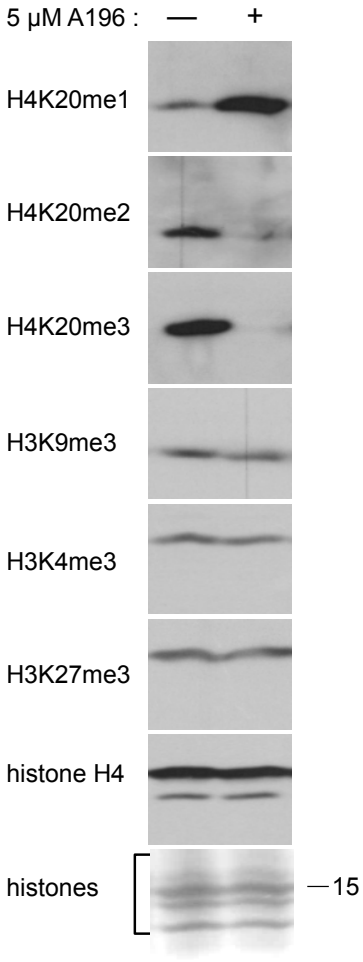
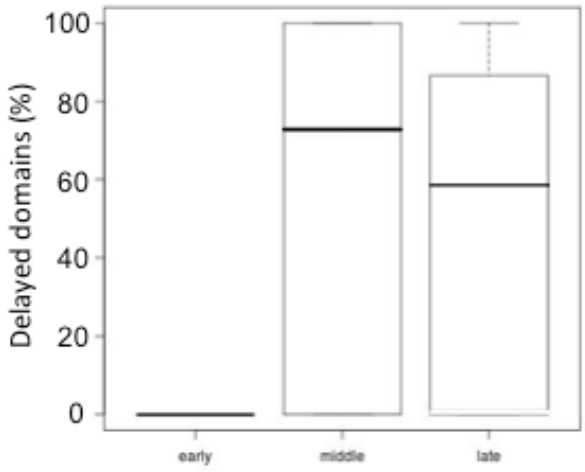
Appendix. Brustel J et al. Histone H4K20 tri-methylation at late-firing origins ensure stimely heterochromatin replication.



Appendix Figure S1: PR-Set7 depleted U2OS cells accumulate in S-phase, indicating improper DNA replication. Cell-cycle analysis by FACS showing DNA content (7AAD, x-axis) versus cell number (left panel, y-axis) or versus BrdU incorporation (right panel, y-axis) three days after control (luciferease) and PR-Set7 shRNA expression. DNA replication was measured by treating control and PR-Set7 shRNA expressing cells with BrdU for 2h, followed by BrdU-specific and FITC-conjugated antibodies.



Appendix Figure S2 : Immunoblot analysis of H4K20me1, H4K20me2, and H4K20me3, H3K9me3, H3K4me3, H3K27me3 and histone H4 levels and SDS-page analysis of total histone levels (Coomassie staining) in EBNA1-expressing HEK293 cells untreated or treated with A-196 inhibitor during 6 days.



Appendix Figure S3 : Box-plot showing the percentage of delayed domains in each timing category in immortalized MEFs treated with shRNA (2) ORCA. Note the high percentage of delayed domains in mid and late categories, indicating a broader effect of ORCA depletion on replication timing program than the loss of Suv4-20h and H4K20 methylation in MEFs.

coverage genes			
T-test	t	degree of freedom	p_value
delayed vs early	-14.928	163.04	$<2.2^{16}$
delayed vs middle	-12.765	305.76	$<2.2^{16}$
delayed vs late	-7.8907	199.44	1.949E-13
delayed vs stocha	-6.0761	115.14	1.63E-8

coverage H3K27ac			
T-test	t	degree of freedom	p_value
delayed vs early	-44.942	770.77	$<2.2^{16}$
delayed vs middle	-14.042	783.05	$<2.2^{16}$
delayed vs late	-9.1731	596.31	$<2.2^{16}$
delayed vs stocha	-31.721	141.55	$<2.2^{16}$

coverage H3K9me2			
T-test	t	degree of freedom	p_value
delayed vs early	13.518	130.55	$<2.2^{-16}$
delayed vs middle	-14.042	167.92	1.521E-015
delayed vs late	8.4551	139.17	3.39E-13
delayed vs stocha	11.321	114.18	$<2.2^{-16}$

Appendix Table S1 : Values of different T-test associated with the figures 4E, 4F, and 4G. The table shows are the statistical value of the T-test (t), the degree of freedom and the p-value. T-test were performed with R program version 3.2.3

ORIs	start	end	ORI location	primers	
ORI1-ND	82505263	82505426	chr11:82505263-82505426	CCTAAACCTGCTCTGCTAAGG	GGCACAGATAGGTCAACACA
ORI2-ND	82646414	82646774	chr11:82646414-82646774	CCATGCCAAGGTATCTCTTCTC	CTCTCCCTCTCTCTGTCTTTCT
ORI3-ND	82684108	82684567	chr11:82684108-82684567	ATCCCAAACTTCCCATCTAACC	GGAAGGATGCATTTCTGTAGGA
ORI4-ND	82685667	82686187	chr11:82685667-82686187	CCTTTCTCCTGCCTGCTTATT	GACAATGTTGGTGCGTCAAAG
ORI5-ND	71274933	71275606	chr11:71274933-71275606	GAGGGTGAGGAAAGAGACAAATAG	TTCTCCCATCCATTGTGCTTAG
ORI6-ND	71243973	71244400	chr11:71243973-71244400	CAGAGGTGGTAACCTCAGAAAG	TGGCAAGACAGAAGAGATGAAG
ORI7-ND	71196358	71199261	chr11:71196358-71199261	CGAGCGGAAGGAGCTTAATAAT	GTGACAGTTTGCCTCTGATCT
ORI8-ND	67017089	67017576	chr11:67017089-67017576	CAGCATCAGCCATGTTCTTTG	GTGTACTCCTTGGCTGTCTTAG
ORI9-ND	66958207	66958903	chr11:66958207-66958903	TTTCAGCTGGATCCCAATAGAC	GAACCCACAAACTCGAGATGA
ORI10-ND	66775679	66776129	chr11:66775679-66776129	GCAGTGTGGATGAAATGATCTG	GTGTCTTTGCCCTCCTACTT
ORI11-ND	66519315	66519914	chr11:66519315-66519914	CCTGTACCTGGCATGGTTT	GCATAGCACCGTATCCCTAATG
ORI12-ND	65981038	65981586	chr11:65981038-65981586	ACCAAATCGCCTCCTTCTAAAC	CAAGGGCCAAGGACAAGAAA
ORI13-ND	65849784	65850098	chr11:65849784-65850098	GCACACTTCCTCCCTCATAAA	GTCTGTCTCACATTCGATCTCC
ORI14-ND	64878087	64878780	chr11:64878087-64878780	TCCCAGAGTCTCCTGTTGATAC	CCTGTCCTTCCGTGCAAATAA
ORI1	91364840	91365042	chr11:91365072-91367583	CTTACACAGCAAGCCCTAGAC	AGCAACATAGACCACCTTCAC
ORI2	91473274	91473511	chr11:91472486-91474388	AGGCAGTGCTAAGTGTTATGG	CACAGCCTTCTGACCTGTTTA
ORI3	91998063	91998271	chr11:91997085-91999288	CTGGGAGGAGATGAACTTAGAC	CATTCACTGCCTCTCCATCT
ORI4	92655589	92655885	chr11:92655064-92656805	CAGCTGGGAGTGTCCATTTAG	TGACTGGGAAGGGACTTGTA
ORI5	66782899	66783024	chr12:66782899-66783024	GTATGAGTGGCAGAGAGAATCG	AGTCAACCACCGAACATATCC
ORI6	39984698	39985795	chr17:39984698-39985795	CAGCTGGGAGTGTCCATTTAG	TGACTGGGAAGGGACTTGTA
ORI7	21419636	21419959	chr18:21419636-21419959	CGGCAAGCCAGAGAACAATA	GGTAGCATGTCACCCTAATCA
ORI8	114481978	114482092	chr7:114481978-114482092	TGAAGCGTCTGATGTGTCTG	TCCGATTGCCTTCTGGATAAC
ORI9	114482109	114482238	chr7:114482109-114482238	GGGAACCCATTAGGAAGCTATT	AGATGATCCACCTCATCCTAGA
ORI10	86061118	86061443	chr13:86061118-86061443	CTCACCCACGCACAATGATA	GAACTGACGGTATGCTAGAAGG
ORI11	21106191	21106716	chr18:21106191-21106716	GCTATGGGTTTGGCGATTTAC	CCTGGGTGTGATCCAAGAAA
ORI12	27074390	27074715	chr6:27074390-27074715	TGAACTGGTTGCATTGCTTATG	GCTATGTTGCTCCTCCCATTA
ORI13	64963191	64963691	chr6:64963191-64963691	ACAGTGTCAGAACACCCAAG	GGTGTCACTCGTTCTCTGAATAG
ORI14	67147836	67148711	chr7:67147836-67148711	CAACACCGCAGAAATGACAAG	CGCCATTGTGAGGATCTGTAA
ORI CTRL	618166046	61816497	chr15:6181660461816497	TTCTGTTTTCCCCAGCCTTA	TCGGCTGAACTGTGTTCTTG
ORI CTRL	82131951	82131972	chr11:8213195182131972-	CTCGCCTTTCTCATGGATTCAT	GGACAGTGTCAGTTACGGAAGGT

Appendix Table S2 : coordinates in mm9 and primer sequences used to analyze the different late-firing replication origins in MEF models.

Supplementary Materials and Methods:

Detailed ChIP-qPCR experiments. ChIP experiments with MEFs$^{364.2}$ were performed as described previously (Tardat et al., 2010). Cells were fixed with 1% formaldehyde (10 min) and quenching was done with 125 mM Glycine. After a PBS wash, cells were resuspended in lysis buffer (10min, 4°C). After sonication with a vibra Cell (Bioblock) to obtain chromatin fragments less than 800 bp, ChIP was performed with 200 μg of sheared chromatin incubated with protein G magnetic beads (Invitrogen) coupled with the appropriate antibody as follows: anti-GAL4 (Santa Cruz), anti-Flag M2 (Sigma), anti-H4K20me1 (Diagenode) and anti-H4K20me3 (Abcam). ChIP experiments were performed at least three times from independent chromatin preparations and quantitative PCR analyses of ChIP DNAs were performed using a SYBR green quantitative PCR kit (Invitrogen) and a light cycler 480 II (Roche) under conditions standardized for each primer set. The amount of DNA in ChIP samples was extrapolated from standard curve analysis of chromatin DNA before immunoprecipitation (input) and values were represented as a percentage of input chromatin.

CHIP-qPCR on EBV episomes was performed with transfected HEK293 EBNA1^{+} cell lines. Cells were resuspended in PBS (per 2 x 10^7 cells) and fixed with 1% of methanol-free formaldehyde (Thermo Scientific) for 5 minutes on a roller at room temperature. The cross-linking reaction was then quenched with glycine (1.25 M) and incubated for another minute on the roller. After washing once with PBS and once with PBS 0,5% NP-40, cells were resuspended in PBS containing 10% glycerol, pelleted and snap frozen in liquid nitrogen. For plasmid ChIP analysis, cells were thawed on ice and resuspended for lysis in LB3+ buffer (25 mM Hepes (pH 7,5), 140 mM NaCl, 1 mM EDTA, 0,5 mM EGTA, 0,5% Sarcosyl, 0,1% DOC, 0,25% Triton-X-100, containing protease inhibitor cocktail (Roche)) to a final concentration of $2x10^7$ cells/ ml. Sonication was performed for 20 min using the Covaris S220 with the settings 150W, 200 cycles/burst, 20% duty cycle at an average temperature of 5°C. After sonication,

sheared chromatin was pre-cleared with protein A beads for 2h, and incubated 16h at 4°C, with the appropriate antibody: anti-GAL4 (Santa Cruz Biotechnologies, sc-577), anti-H4K20me1 (Diagenode, MAb-147-100), anti-H4K20me3 (Diagenode, pAB-057-050), anti-MCM3 (Ritzi et al., 2003). BSA-blocked protein A beads were then added and incubated for at least 4h, and then sequentially washed with RIPA-150 mM NaCl, RIPA-300 mM NaCl, LiCl buffer (250 mM LiCl, 0.1% SDS, 0,5% DOC, 1% NP-40, 50 mM Tris (pH 8.0) and finally twice in TE (pH 8.0) buffer. Immunoprecipitated chromatin fragments were eluted from the beads by shaking for 10 min at 65°C with 100μl of TE and 1% SDS. The elution was treated with RNAse A for 2h at 37°C and with proteinase K at 65°C for 16h. DNA was purified using the NucleoSpin Extract II Kit according to manufacturer's instructions. Quantitative PCR analysis was performed using the Roche LightCycler 480 System and the SYBR Green I Master (Roche) according to the manufacturers instructions. Amplification was performed using the standard program and an annealing temperature of 60°C. The primer pairs for FR, UAS and ORI-RDH sequences are available in supplementary Table 1. Quantitative PCR values were calculated as described above and represented as fold enrichment relative to isotype IgG control.

Detailed Replication timing analysis. 30 x 10^6 MEFs$^{364.2}$, untreated and 4OHT-treated, were incubated with BrdU for 2 hours before ethanol fixation. After centrifugation, fixed cells were suspended in DPBS with RNAse A (0.5 mg/ml) and propidium iodide (50 μg/ml) for 30 min at room temperature. 100,000 cells were sorted into two fractions, S1 and S2, using INFLUX 500 (Cytopeia BD Biosciences) corresponding to Early and Late S-phase fractions respectively. Cells in each fraction were suspended in a lysis buffer (50mM Tris pH=8, 10mM EDTA, 300mM NaCl, 0.5% SDS, and 0.2mg/ml of Proteinase K) and neo-synthesized DNA was immuno-precipitated with BrdU antibodies (Anti-BrdU Pure, BD Biosciences, # 347580). The quality of enrichment of early and late fractions in S1 and S2 was performed by qPCR with CAV2 oligonucleotides (early control) and with bgGRM8 oligonucleotides (late control) as

described previously (Hadjadj et al., 2016). Whole genome amplification was conducted (WGA, Sigma) to obtain the amount of DNA (500 ng) required for microarray hybridization. To verify that this step did not introduce bias, a post WGA qPCR was performed to confirm the specific enrichment in both the S1 and S2 fractions. After amplification, early and late neo-synthesized DNAs were labeled with Cy3 and Cy5 ULS molecules (Genomic DNA labeling Kit, Agilent) as recommended by the manufacturer. The hybridization was performed according to the manufacturer's instructions on 4×180K mouse microarrays (SurePrint G3 Mouse CGH Microarray Kit, 4x180K, AGILENT Technologies, reference genome: mm9) that covers the whole genome with one probe every 13Kb (11 Kb in RefSeq sequences). Microarrays were scanned with an Agilent High-Resolution C Scanner using a resolution of 2 μm and the autofocus option. Feature extraction was performed with the Feature Extraction 9.1 software (Agilent technologies). Analysis was performed with the Agilent Genomic Workbench 5.0 software. The $\log_2$-ratio timing profiles were smoothed using the Agilent Genomic Workbench 5.0 software with the Triangular Moving Average option (500 kb windows).

To determine the replication domains in different conditions, algorithms from CGH applications in the Agilent Genomic Workbench 5.0 software were used, particularly the aberration detection algorithms (Z-score with a threshold of 1.8) that define the boundaries and magnitudes of the regions of DNA loss or gain corresponding to the late and early replicating domains respectively. Then, a comparative analysis of replication domains was performed between the different cellular conditions, in order to determine DNA segments with significant replication timing changes. A Student test (T-Test) was performed on the average of the Log^2 values of every domains with R program 3.2.3 and significant difference is annotated when p-value < 10^{-3}. The intersection with different data sets was performed with GALAXY tools and T-test was performed to identify significant differences. The microarray data have been deposited in the Gene Expression Omnibus (GEO) (accession no. GSE69084). Positions of genes used for gene coverage come from RefSeq mm9. Positions of H3K27ac peaks used for

coverage come from GSM1631248 (GEO database). Positions of H3K9me2 peaks used for coverage come from GSM887877 (GEO database).

Plasmid replication assay. GFP-positive reporter plasmids (1μg) were transfected into HEK293 EBNA1$^+$ cell line stably expressing the respective GAL4-fusion protein using Lipofectamine2000 (Life technologies) according to manufacturers instructions. Transfections with comparable efficiencies were verified by visualizing GFP-positive cells. Six days post-transfection, cells were harvested according to the HIRT protocol. After washing with PBS, cells were first equilibrated in 10 ml of TEN buffer (10 mM Tris-HCl pH 7,5, 1 mM EDTA, 150 mM NaCl). Cells were then diluted in 1.5 ml of TEN buffer and an equal volume of 2xHIRT buffer (1,2% SDS, 20 mM Tris-HCl pH 7,5, 20 mM EDTA) was then added for cell lysis. The lysate was then incubated at 4°C for 16h, in the presence of 1.25 M NaCl. After centrifugation (2000xg) for 1h at 4°C, DNA was purified by phenol-chloroform extraction and digested with 40 U *Dpn*I (NEB) in presence of RNase (Roche). Digested DNA (300 ng) was electroporated into Electromax DH10B competent cells (Invitrogen) and ampicillin-resistant colonies, representing the number of recovered plasmids, were counted. The FR-DS plasmid was always transfected in parallel and the number of resulting colonies was used for normalization. Statistical analysis of replication efficiency was performed using paired students T-test.